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The extraction and mechanism of a novel isomaltulose-synthesizing enzyme from *Erwinia rhapontici*

Peter S. J. CHEETHAM
Tate and Lyle Group Research and Development, Philip Lyle Memorial Research Laboratory, P.O. Box 68, Whiteknights, Reading RG6 2BX, U.K.

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The single enzyme that mediates the bioconversion is demonstrated to be located in the cells' periplasmic space, a site that facilitates its use as an industrial biocatalyst, and to be a previously undescribed hexosyltransferase with four novel features. The enzyme is sucrose-specific, and has an intramoleular mechanism in which both glucose and fructose residues appear to be enzyme-bound. Thirdly, it is reaction-non-selective, forming simultaneously isomaltulose and a second hitherto uncharacterized α-(1→1)-linked disaccharide (trehalulose), by hydrolysis of sucrose followed by reaction of glucose with the C-6 and C-1 positions of the fructofuranose respectively. Finally, on extended incubation an unusual recycling mechanism caused the concentration of isomaltulose, the kinetically preferred product, to reach a transient maximum concentration and then fall, and the concentration of trehalulose, the thermodynamically favoured product, to rise slowly.

Isomaltulose (6-*O*-α-D-glucopyranosyl-D-fructofuranase), a functional isomer of sucrose commonly referred to as palatinose, is a reducing disaccharide (60% of glucose), of about one-third the sweetness of sucrose, that is very resistant to hydrolysis by acid and invertase (β-D-fructofurnaosidase). It has very similar physical properties to sucrose, is found naturally in small quantities in honey (Siddiqui & Furgala, 1967) and has potential industrial uses either as itself or in a hydrogenated form (Bucke & Cheetham, 1981, 1982; Cheetham *et al.*, 1982; Munir, 1982; Kutzbach *et al.*, 1982; Shimizu *et al.*, 1982; Takazeo *et al.*, 1982; Lanterno, 1983; see also British Patent Specification 1 429 334 assigned to the South German Sugar Co.).

Historically the first mention of isomaltulose derived from a microbial source was by Stodola *et al.* (1952) and Sharpe *et al.* (1954), who described it as a by-product of leucrose (5-*O*-α-D-glucopyranosyl-D-fructopyranose) and dextran formation by *Leuconostoc mesenteroides*, although the formation of reducing disaccharides by the action of this organism, and by *Streptococcus bovis* acting on sucrose, was first mentioned by Hehre (1951). Bailey & Bourne (1959) later noted that a reducing disaccharide similar to isomaltulose was produced by *Saccharomyces bovis*, and Stodola *et al.* (1956) and Sharpe *et al.* (1960) confirmed the formation of isomaltulose by enzymes derived from *L. mesenteroides*. Later an α-glucosidase from *Saccharomyces italicus* was found to form isomaltulose as a transglucosylation product (Halvorsen, 1966). More recently, Fuji *et al.* (1983) have studied the oligosaccharides formed by the action of *Serratia plymuthica* on sucrose. They found isomaltulose to be the major product and the other sugars to be 1-*O*-α-D-glucosylfructose, isomaltose and isomelezitose (6^F-α-D-glucosylsucrose).

Isomaltulose can be produced in high yields by using very stable continuous columns of immobilized *Erwinia rhapontici* (Cheetham *et al.*, 1982; Bucke & Cheetham, 1982). The enzyme activity was stabilized by immobilizing in alginate rather than other support materials, by using structurally intact but non-growing cells rather than isolated enzyme, disrupted cells or growing cells, by using concentrated pure sucrose as substrate and by maintaining complete conversion of the sucrose into isomaltulose. The present study demonstrates the value of investigating the inter-relationships between the physiological state of the cells, conditions of immobilization and use, and the activity and stability of the immobilized cells.

The present paper describes the enzyme that is responsible for this microbial bioconversion,

which is an example of the growing exploitation of microbial enzymes in the form of immobilized cells (Abbott, 1977; Cheetham, 1980), the most important of which is glucose isomerase (Bucke, 1983).

Methods

Erwina rhapontici (N.C.P.P.B. 1578) cells were grown, viable cell counts were made and enzyme activity was assayed at its optimal pH of 7.0, its optimal temperature of 30°C and with a substrate concentration of 1.6M as described previously (Cheetham *et al.*, 1982). In high-pressure-liquid-chromatographic analysis the assumption was made that sucrose, isomaltulose and trehalulose solutions of the same concentration have the same refractive index. Cells were osmotically shocked by resuspending 1.5g wet wt. of cells in 150ml of ice-cold deionized water for 30min. Cells were sonicated as a 10% (wet wt./vol.) suspension in deionized water at 2.8A for 340min with the use of a probe with a tip diameter of 2.5cm and cooling the sample at 5min intervals. Cells were disrupted by mixing 2g wet wt. of cells with 2g of dry sand and 2ml of deionized water and shaking at maximum amplitude for 20min at room temperature in a Mickle shaker (Laboratory Engineering Co., Gomshall, Surrey, U.K.). Cells were treated with detergent by resuspending 0.5g in 10ml of 0.15% (w/v) Triton X-100 solution for 3h. Cells were shocked at 70°C for 3min in a water bath or by adjusting to pH2.0 or pH10.0 with acid or alkali before being adjusted back to neutral pH. After each treatment the cell suspension was centrifuged at 17000*g* for 30min at 30°C, and the pellet of cells or cell debris and the supernatant were assayed.

In the radiometric assays enzyme was obtained and assayed at 30°C with 1.6M-sucrose, pH7.0, as described above except that unlabelled glucose or fructose (both 0.5M) and very small quantities of radioactively labelled sucrose, glucose and/or fructose (Amersham International, Amersham, Bucks., U.K.) were added to give working radioactivities of 10μCi/ml. Then 2μl samples of solutions diluted to 2% (w/v) were chromatographed on Merck Silica gel 60 plates in butanol/methanol/water (5:3:2, by vol.) for 16h. The plates were then incubated in contact with X-ray film (Kodak, Hemel Hempstead, Herts., U.K.) for several days before developing. The original or a duplicate plate was then stained for free or combined fructose with carbazole stain (Adachi, 1965). Results could be quantified by scraping off each radioactive spot and counting its radioactivity in a toluene-based scintillant containing 2,5-diphenyloxazole (4g/l), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.1g/l) and Fison's mix no. 1 emulsifier with the use of a 10000c.p.m. external standard.

The experiments described above that utilize labelled sugars do not allow the usual procedures for determining whether the reaction proceeds via an odd or even number of steps depending on whether the glycosidic bond of the product is inverted or not with respect to the substrate. This is because sucrose can be regarded both as β-D-fructofuranosyl-α-D-glucopyranose and as α-D-glucopyranosyl-β-D-fructofuranose, and so is hydrolysed by both invertases and α-glucosidases.

Measurement of the isomaltulose/trehalulose ratio is one of the few methods of directly determining the reaction mechanism. This is because it is not possible to use standard methods (Fersht, 1977). Measurement of the ratios of the products produced from different substrates cannot be carried out since the enzyme is substrate-specific. Moreover, measurement of the rates of formation of the two products with various ratios of acceptor to donor molecules cannot be employed since no equilibration of exogenous monosaccharides with the reactants inside the active site take place (Fig. 1). Furthermore, this reaction is not amenable to spectrophotometric detection or to ^{13}C-n.m.r. analysis because isomerization of the fructose should take place over the same time periods that are required for structural analysis.

Results

Extraction of the enzyme

Enzyme could be extracted by osmotic shocking (Table 1), together with periplasmic marker enzymes such as acid phosphatase (35μM-*p*-nitrophenyl phosphate hydrolysed/min per ml of extract), but without any significant cytosolic enzymes. Other methods of extraction were less successful (Table 1), and permeabilization increased activity only slightly (latency 10–15%). No sucrose or isomaltulose could be detected in the contents of the cells as liberated by Mickle-shaking or sonication, further indicating that the enzyme that forms isomaltulose is not located intracellularly and showing that isomaltulose does not function as a compatible solute as in *Dunaliella* species (Brown, 1974). The amount of enzyme solubilized was not related to the degree of cell disruption as measured by the viable cell count or to the amounts of protein released, and could not be enhanced by sonication or detergent treatment of the cell debris producing by milling. The soluble extract obtained by osmotic shocking contained ten protein bands on polyacrylamide-gel electrophoresis.

Further proof for a periplasmic rather than an

Table 1. *Extraction and location of a soluble isomaltulose-synthesizing enzyme from E. rhapontici (N.C.P.P.B. 1578)*
For full experimental details see the text. N.D., Not determined.

	Activity of cell debris (μmol of isomaltulose/min per g wet wt. of cells)	Activity of cell extract (μmol of isomaltulose/min per ml of extract)	Concn. of protein in cell extract (mg/ml)	Specific activity (μmol of isomaltulose/min per mg of protein)	Viable cell count (colonies/g wet wt. of cells)
Intact cells	59.94 (intact cells)	–	–	–	2.2×10^9
Mickle-shaken cells	27.63	4.58	32.6	0.14	6.6×10^7
Osmotically shocked cells	22.66	0.12	3.3	4.11	8.2×10^8
Sonicated cells	56.53	1.12	14.9	0.14	N.D.
Detergent-treated cells	1.16	0.023	N.D.	N.D.	4.6×10^8

intracellular location was provided by the fact that addition of a non-lethal dose of the antibiotic lincomycin (10mg/l) to the growth medium enhanced the cells' activity by 60%. Lincomycin is known to inhibit proteinases that limit the synthesis or degrade other periplasmic proteins (Levner *et al.*, 1977).

Properties of the enzyme

The enzyme had no ion or cofactor requirements, and possible intermediates such as UDP-glucose or polysaccharide were not observed. It is not a phosphorylase, as no sugar phosphates could be detected and sucrose was not rapidly hydrolysed in the presence of enzyme and arsenate. No evidence for a regenerated co-substrate was found, as in some transferases (EC 2.7.5. group). The enzyme is absolutely substrate-specific towards sucrose (K_m 0.28M), since a variety of sugars including maltose, lactose, raffinose, galactose, arabinose and mannose proved to be completely unreactive when tested alone or with sucrose. Such absolute substrate-specificity for both donor and acceptor is surprising, because sucrose is a small molecule and because other isomaltulose-producing organisms readily use mannose, arabinose and glucose as acceptors (Mauch & Schmidt-Berg-Lorenz, 1964). Furthermore glucosyltransferases are usually substrate-non-specific and use fructose only when it is present in high concentrations relative to the preferred acceptor. The reaction is essentially irreversible (less than 0.03M-sucrose remaining), indicating a free energy of about −11.8kJ/mol compared with a ΔG value of −28kJ/mol for the hydrolysis of sucrose. The activation and enthalpy of the reaction are calculated to be 17.00 and 14.5kJ/mol respectively.

Mechanism of the enzyme

The enzyme converted labelled sucrose into isomaltulose (approx. 85% yield). Isomaltulose formed from specifically labelled sucrose (samples 5–8, Fig. 1) could be hydrolysed with acid to yield glucose and labelled fructose. The labelled or non-radioactive isomaltulose or trehalulose prepared as above could also be purified by chromatography on a 115cm × 2.2cm diam. column of Dowex AGW X40 (200–400 mesh; K^+ form), or by preparative high-pressure liquid chromatography with a Waters Prep LC/system 500A, with four PrepPak 500/C18 columns used in series, both with water as the eluate. Purified isomaltulose and trehalulose formed from universally or specifically labelled sucrose had very similar specific radioactivities. Smaller concentrations of a second disaccharide were also formed (Fig. 1), for which the structures 1-*O*-α-D-glucosylpyranosyl-β-D-fructopyranose

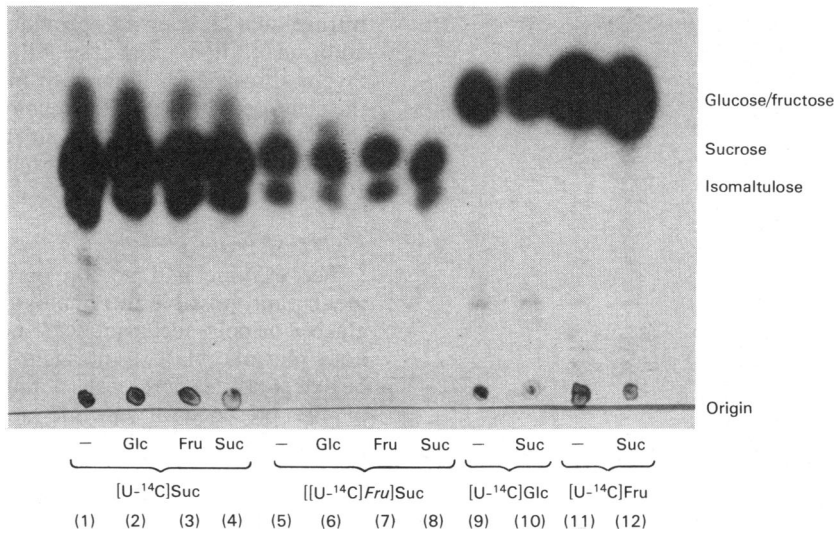
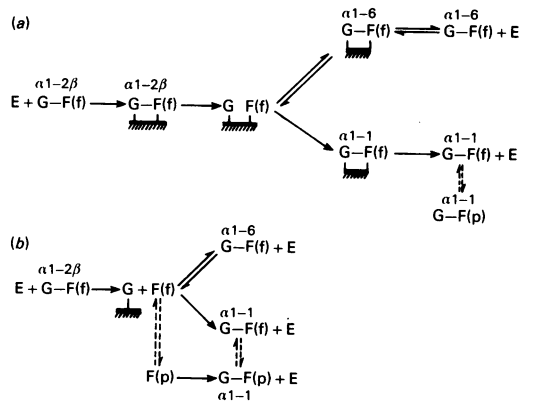


Fig. 1. *Autoradiography of the products of the reaction of radioactively labelled sugars with soluble enzyme obtained from osmotically shocked E. rhapontici cells*

The samples illustrated are (left to right) enzyme incubated with [U-^{14}C]sucrose alone (1), [U-^{14}C]sucrose supplemented with glucose or fructose (2 and 3 respectively) and non-radioactive sucrose (4). Samples (5)–(8) are sucrose specifically labelled in the [U-^{14}C]fructose moiety supplemented as in samples (1)–(4). Samples 9 and 10 are [U-^{14}C]-glucose incubated alone and supplemented with non-radioactive sucrose, and samples (11) and (12) are [U-^{14}C]-fructose incubated alone and supplemented with non-radioactive sucrose. Because of the relatively short incubation times used in this experiment, the amounts of trehalulose formed were insufficient to be easily detected visually as in Fig. 2, but could be easily quantified by scintillation counting as described in the Methods section.

(major form, 66%) and 1-*O*-α-D-glucosylpyranosyl-β-D-fructofuranose (33%) were determined by ^{13}C n.m.r. The neologism trehalulose is proposed, by analogy with lactose/lactulose, for this new example of a (1→1)-linked disaccharide. Little free glucose or fructose (0.07M and 0.10M respectively) and no oligosaccharides were formed (Fig. 1), showing that water and the disaccharide products are poor acceptors. Isomaltulose could not be converted non-enzymically into trehalulose, and vice versa, and no sucrose was formed when either purified isomaltulose or trehalulose was incubated with enzyme.

An intramolecular (direct or monomolecular) transglycosylation mechanism, with both disaccharides being formed via a common intermediate, is presumed. The reaction is not mediated by closely associated but distinct enzymes, or even by one protein possessing two active sites with different specificities (Yourno *et al.*, 1970), because little monosaccharide is formed, because exogenous labelled monosaccharides are not incorporated into either disaccharide product whether provided alone or with sucrose (Fig. 1), and because these monosaccharides did not affect the rates of product formation and sucrose consumption. An enzyme-linked glucose intermediate is indicated, since if free glucose were present sugars such as trehalose, isomaltose, maltose, maltulose and maltotriose could be formed. Similar logic implicated the involvement of enzyme-linked fructose (Scheme 1*a*). Furthermore the enzyme's absolute substrate-specificity and its non-reaction



Scheme 1. *Proposed mechanisms for the formation of isomaltulose and trehalulose from sucrose by the soluble enzyme from E. rhapontici*

Key: G, glucopyranose; F(f), fructofuranose; F(p), fructopyranose; E or ////////, enzyme. Enzyme-catalysed reactions are denoted by continuous lines and non-enzymic isomerization by broken lines. The direction of reaction is roughly indicated by the relative thicknesses of the lines. Note that at equilibrium both free fructose (Furcke *et al.*, 1979) and the fructose component of trehalulose exist predominantly (approx. 66%) in the pyranose form.

with and non-production of monosaccharides also implies multiple attachment of the substrate to the enzyme (Scheme 1*a*).

The following mechanism is proposed, involving binding of sucrose to the enzyme, its hydrolysis and then the synthesis of α-(1→6)- or α-(1→1)-bonds by non-selective reaction of glucose with the C-6 and C-1 secondary hydroxy groups of the fructofuranose (Scheme 1*a*). Surprisingly, isomaltulose formation requires rotation of the fructose residue through 180°, whereas trehalulose, which is formed in much lower yields, requires no such rotation and is presumably formed by reaction before the enzyme-linked fructose residue can rotate. Either the glucose or fructose moiety can be regarded as the leaving group.

A less likely alternative mechanism involving only enzyme-bound glucose can also be envisaged (Scheme 1*b*) in which reaction of glucose with both the furanose and pyranose forms of fructose may take place. However, attempts to channel exclusively the 6′-blocked substrate 6′-chlorosucrose into trehalulose were unsuccessful. Also, borate (0.3M), which should selectively form a complex with the free fructofuranose, did not inactivate the enzymes, unlike other isomaltulose-forming enzymes (Mauch & Schmidt-Berg-Lorenz, 1964). Isomerization to fructopyranose with subsequent reaction to trehalulose (Scheme 1*b*) is unlikely, since glycosylation should occur before mutarotation can occur, since no β-linked disaccharides such as gentiobiose are formed, and also before fructose can diffuse out of the active site, as little fructose is formed. Perhaps the fructose is held in the furanose configuration by association with reactive amino acids in the active site of the enzyme. Also, unless isomerization takes place much more rapidly in the active site than in free solution, the ratio of isomaltulose to trehalulose formed should vary during the reaction (Fig. 2). In addition, reaction via open-chain fructose is unlikely, as n.m.r. shows that very little is present,

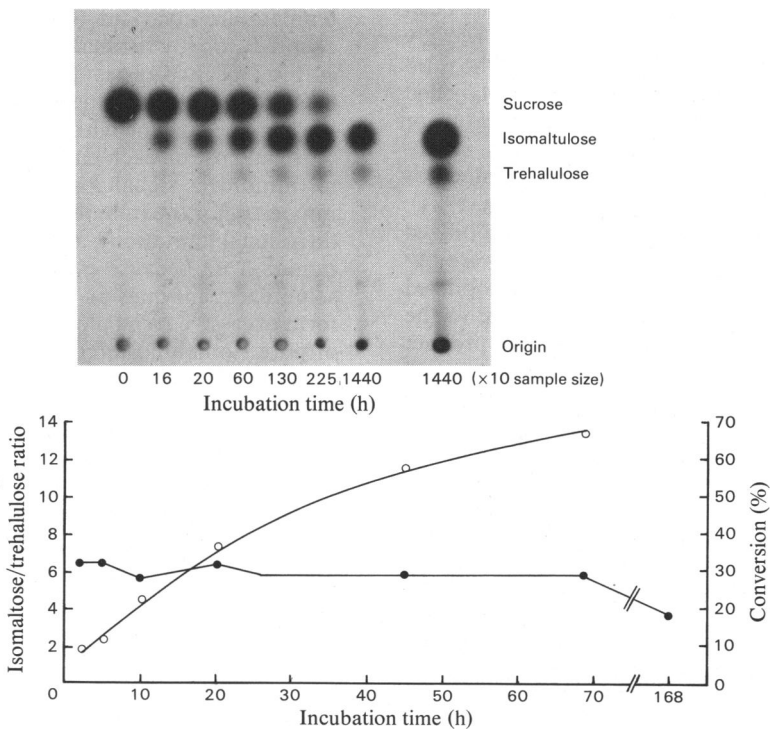


Fig. 2. *Measurement of the ratio of isomaltulose to trehalulose* (●) *formed during a typical reaction of the soluble enzyme from E. rhapontici with sucrose*

The percentage conversion of sucrose into products is also depicted (○). Thus 1% conversion of the 1.6M-sucrose used as the substrate in this experiment results in the generation of approx. 0.0139M-isomaltulose and 0.0021M-trehalulose. Extraction and assay of samples and immobilization of enzymes and cells were as described in Table 1 and Cheetham *et al.* (1982). Isomaltulose and trehalulose were determined either by high-pressure liquid chromatography or by t.l.c. (see inset) followed by scraping the radioactively labelled spots off the t.l.c plate and counting their radioactivity.

and also the reaction was not inhibited by xylitol or mannitol (up to 0.25M), which should form complexes with acyclic fructose (Ananichev *et al.*, 1980).

Reaction non-selectivity

A surprising feature was the simultaneous rather than sequential formation of two disaccharides from pure sucrose (Fig. 2). Simultaneous formation is an intrinsic property of the enzyme, since the ratio of isomaltulose to trehalulose produced was constant throughout the early stages of the reaction and under a variety of conditions (Fig. 2), whereas in a sequential reaction an early peak in the concentration of the intermediate should be apparent. Very similar isomaltulose/trehalulose ratios, indicating that isomaltulose and trehalulose are formed in parallel, were obtained when antimicrobial agents such as chloramphenicol or benzylpenicillin were added, proving that trehalulose is not formed by the action of contaminant micro-organisms, with samples of cells harvested during growth phase, intact non-growing cells, disrupted cells, partially purified enzyme or cells that had been pH- or temperature-shocked, and under a variety of experimental conditions. For instance, the ratio did not vary significantly, when pulses of labelled sucrose were supplied, with the pH, temperature or substrate concentration used, when cells or enzyme were immobilized by a variety of methods (Cheetham *et al.*, 1982; Bucke & Cheetham, 1981), or throughout the useful life of the immobilized cells, with the proviso that lower values were observed in batch reactions and when immobilized biocatalysts were used, presumably owing to the influence of diffusional restrictions on product transfer. Also, under the conditions found optimal for *E. rhapontici*, immobilized *Erwinia carotovora* cells formed roughly equal proportions of trehalulose and isomaltulose throughout its half-life (975h), the cells having an initial activity of 5.85μmol of trehalulose formed/min per g wet wt. of cells.

Note also that other isomaltulose-producing organisms also form trehalulose (Mauch & Schmidt-Berg-Lotenz, 1964; Kutzbach *et al.*, 1982; Munir, 1982; Shimizu *et al.*, 1982), and an even more marked non-selectivity is probably exhibited by *E. carotovora* (Lund & Wyatt, 1973).

Changes in product concentrations after prolonged incubations

On extended incubation, once all the sucrose had been consumed a very slow but continuous decline in the initial concentration of isomaltulose and corresponding increases in that of trehalulose occurred (Fig. 2). In an extreme case, in which flow through a column of immobilized cells was stopped for 10 days, the isomaltulose/trehalulose ratio in the syrup fell to 1.15:1 (0.83M and 0.72M respectively). This low rate of reaction is consistent with the observation that isomaltulose is much more stable to acidic hydrolysis than is sucrose. Confirmation was provided by the very slow formation of trehalulose by the enzyme working 'in reverse' when the enzyme or cells were incubated with purified isomaltulose, whereas pure trehalulose proved to be completely inert over the time periods used in these experiments. Thus isomaltulose is metastable and its formation is slowly reversible, whereas trehalulose is formed virtually irreversibly (Fig. 2), perhaps because it is not re-admitted back into the active site.

Virtually irreversible enzymes, such as many enzymes with a regulatory role in metabolism, are usually characterized by a relatively large ΔG value. Thus the low ΔG of about -12kJ/mol calculated for isomaltulose synthase and the observation that the heats of hydrolysis of both disaccharides are both small because yields are temperature-invariant (4–65°C) can only be accounted for by isomaltulose having a greater entropy of hydrolysis than sucrose. Thus over long time periods isomaltulose is slowly recycled through the enzyme with a proportion sequestered as trehalulose on each passage (Scheme 1). In order to minimize trehalulose formation it is thus important for the immobilized enzyme to have low diffusional limitations, and also to be permanently immobilized because even low concentrations of solubilized enzymes in the column eluate could form excessive trehalulose and decrease the yields of pure isomaltulose recovered by crystallization.

Although little is known about whether the substrate-specificity of enzymes differs when acting in the 'normal' or 'forward' direction and the thermodynamically less favoured 'reverse' direction, it is of note that the enzyme described in the present paper appears to be absolutely substrate-specific for sucrose in the 'forward' direction and for isomaltulose when acting in the 'reverse' direction, trehalulose, the other product of the forward reaction, being unreactive.

Discussion

Many industrially important enzymes have not been subjected to detailed biochemical study. However, in the present paper a new periplasmically located sucrose-specific hexosyltransferase is described that illustrates the advantage of screening for micro-organisms possessing novel

enzymes. Such novel enzymes are especially useful when new products are required.

This periplasmic location is advantageous when the enzyme is to be used associated with its parent cells. This is because, by comparison with intracellular enzymes, it is probably less liable to product inhibition, because substrate transfer and product export are facilitated without deliberate permeabilization and because it will be less subject to physiological turnover mechanisms. Thus for biotechnological applications it may prove to be advantageous to modify the location of normally intracellular proteins by the attachment of appropriate signal sequences (Inouye & Beckwith, 1977; Randall *et al.*, 1978).

This enzyme has a novel intramolecular non-selective mechanism that allows the formation of kinetically or thermodynamically preferred products and has a high activity in a low-water-activity environment, thus allowing a high process intensity consistent with large-scale use to be achieved. The mechanism is similar to the formation of allolactose from lactose (Hall, 1982), although in that case other sugars are also formed by indirect transglucosylation. By contrast, with the *E. rhapontici* enzyme other isomaltulose-producing enzymes readily incorporate monosaccharides (Mauch & Schmidt-Berg-Lorenz, 1964). The enzyme appears to be specific for primary hydroxy groups, catalysing reactions involving the C-6 and C-1 hydroxy groups of fructose, forming isomaltulose and trehalulose respectively. Despite being absolutely substrate-specific, the enzyme is actually reaction-non-selective, non-selectivity being a characteristic of most chemical catalysts but of very few enzymes (Jones *et al.*, 1976; Dixon & Webb, 1979). Two enzymes that have been reported to display this effect, aconitase and alcohol hydrogenase, only catalyse the formation of different constitutional isomers, citrate and isocitrate and axial and equatorial alcohols respectively, presumably because each substrate can bind to the active site in two alternative ways (Bentley, 1969). Also, malate dehydrogenases (EC 1.1.1.38–40) and phosphogluconate dehydrogenase (EC 1.1.1.44) can also act as decarboxylases.

The enzyme bears some similarities to the enzyme (EC 2.4.1.18) that introduces the branches into amylopectin, but can be best classed as an intramolecular isomerase (EC 5.4.–.– group), in which case it would be the first example of such a mutase acting on a sugar. The trivial name isomaltulose synthase is proposed. Alternatively, it could be the first glycosyltransferase (EC 2.4.1.– group) to be shown to have a true intramolecular mechanism.

In many respects isomaltulose would appear to be a secondary metabolite. Trehalulose formation can be regarded as a 'luxury feature' of the enzyme, tolerated because both disaccharides have similar adaptive roles. That is, they enable cells that cannot grow owing to an absence of nitrogenous or other nutrients to sequester sucrose into a carbon and energy store that is unavailable to potential competitors, rather than metabolizing it wastefully and unproductively. Thus it is obviously advantageous for such a process to be mediated by a periplasmically rather than an extracellularly located enzyme. This role is in agreement with the concept that the periplasmic space of Gram-negative bacteria has an organelle-like role possessing specialized metabolic functions and containing enzymes that in Gram-positive organisms are extracellularly located. Thus the isomaltulose synthase described in the present paper can be regarded as a form of extracellular enzyme, although compartmentalized within the outer membrane of the cell.

The marked stabilization of the enzyme by sucrose (Cheetham *et al.*, 1982) is an advantageous feature, as it enables the enzyme to transform efficiently the large quantities of sucrose encountered in the cells' natural environment. This stabilization could also, of course, be due to isomaltulose acting as an inducer of the isomaltulose synthase. This transformation may be a means of the cell avoiding 'substrate-accelerated death' (Dawes, 1976), whereby cell viability is rapidly lost when glucose, for instance, is supplied to a quiescent cell culture. Protection would thus be achieved by maintaining a balanced metabolism by excluding excess carbon source from the cell when nitrogenous and other nutrients are absent, and by providing an energy reserve that prevents the consumption of endogenous metabolic reserves, especially as the long-term survival of cells has been related to a low rate of endogenous metabolism (Dawes, 1976). Another example may be the way in which *Pseudomonas aeruginosa* accumulates gluconate and 2-oxogluconate when exposed to high concentrations of glucose. The enzymes involved, glucose dehydrogenase and gluconate dehydrogenase, are both orientated in the cytoplasmic membrane, so that direct oxidative metabolism takes place in the periplasmic space and most of the products do not enter the cell. Only when glucose is limited does the cell switch its metabolism so that the glucose and 2-oxogluconate are rapidly transported into the cell, phosphorylated and metabolized (Whiting *et al.*, 1976*a*,*b*). Such potentially useful non-growth-associated activities may be best searched for in spores and other forms of resting micro-organisms, which are naturally adapted to environments where nutrients are available in small quantities and/or infrequently.

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